REMARKS

This Reply is responsive to the Office Action dated March 19, 2001. Entry of the foregoing and reconsideration on the merits pursuant to 37 CFR 1.112 is respectfully requested.

The application has been amended as set forth above. In accordance for the new rules for amending applications set forth in 37 CFR 1.121, which took effect on March 1, 2001, a marked up version of the claims showing all amendments is attached hereto as an appendix. Specifically, claim 24 has been canceled and claims 1 and 14 have been amended to indicate that the recited culturing method is performed in the absence of a feeder layer rather than specifying the mere possibility. These amendments find support on page 16, lines 21-23. In addition, claim 1 was amended to delete superfluous language and for grammatical purposes. No new matter was added.

New claims 25-30 were also added by way of amendment above. New claim 25 is similar to pending claim 14 except that it does not require the isolation of EG cells, and it specifies that the method involves the making of germline chimeric avians from primordial germ cells (PGCs) from a Stage XII-XIV avian embryo by introducing the cells into a Stage XII-XIV avian recipient embryo. Support for claim 25 may be found on page 22, lines 10-21, and page 27, lines 10-11. New claim 26 is also similar to pending claim 14 except that it specifies that the method involves the making of either germline or somatic cell chimeric avians from EG cells isolated via primordial germ cells (PGCs) from a Stage XII-XIV avian embryo by introducing the EG cells into a Stage X avian recipient embryo. Support for claim 26 may be found on page 22, lines 10-21.

New claims 27-30 were added to emphasize the novel and unexpected aspects of the invention as identified by the Examiner in the Office Action dated March 19, 2001. Specifically, claim 27 is directed to a method of producing EG cells from Stage XII-XIV avian PGCs, wherein the culturing of the PGCs is performed in the absence of a feeder layer. According to the Office Action, the fact that the culturing method is performed in the absence of a feeder layer is a novel aspect of the invention not seen in the prior art. Support for such a claim is found in original claim 1, at page 15, line 5, and page 16, lines 21- 23.

New claim 28 is directed to an improved method of producing a chimeric avian and like the new claims discussed above, limits the starting PGCs to those derived from a Stage XII-XIV avian embryo. However, this claim leaves out reference to any growth factors in

light of the determination by the Examiner in a related application that the mere fact that the inventors have achieved long term growth of PGCs beyond fourteen days is a novel aspect of the invention. See copending U.S. Serial No. 09/127,624. Finally, new claims 29 and 30 are similar to new claim 28, except that they specify the recipient avian embryo as being either Stage XII-XIV or Stage X, respectively, and further specify that the chimeric avians made by the method are germline, or either germline or somatic cell chimeras, respectively, similar to the strategy set forth in new claims 25-26. Support for claims 28-30 is the same as that found for the new claims discussed above. No new matter has been added.

Turning now to the Office Action, claims 1-24 were rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement for the full scope of the invention. Specifically, while the Examiner believes that the specification enables

(1) a method of isolating avian EG/ES cells using cells from stage X embryos by culturing in the recited growth factors,

(2) a method of making germline chimeric avians using PGCs isolated from the blood of Stage XII-XIV embryos following culturing in the recited growth factors, and

(3) a method of making germline or somatic cell avians using EG cells isolated from cells isolated from Stage XII-XIV embryos following culturing in the recited growth factors, and

(4) an EG/ES cell line made by (1) above,

the Examiner alleges that the specification does not reasonably enable

(a) isolating EG/ES cells from the blood of Stage XII-XIV avian embryos,

(b) using PGCs isolated from the blood of Stage XII-XIV embryos to make either germline or somatic cell embyros,

(c) stably transfecting EG/ES cells or PGCs,

(d) making transgenic avians to isolate proteins or

(e) to isolate phenotypically altered transgenic avians,

(f) EG/ES cells isolated from the blood of Stage XII-XIV embryos,

(g) transfected EG/ES cells, or

(h) the EG cell line P102896 as recited in claim 24.

The allegedly enabled and non-enabled items above will be addressed in turn for the convenience of the Examiner.

First, the Examiner bases the distinction that the specification is enabling for a method of isolating avian EG/ES cells from Stage X embryos but not from Stage XII-XIV embryos on the fact that EG/ES cells from Stage X embryos were allegedly known in the art and shown by the combination of Pain and Simkiss, but that Applicants have not allegedly shown that the cells disclosed in the specification and isolated from Stage XII-XIV embryos are true EG cells. Thus, the Examiner only concedes as enabling methods of making EG cells using the specific growth factor combination and cells from Stage X embryos as known in the art, not Stage XII-XIV as disclosed in the application. The Examiner points particularly to the fact that only germline chimeras using Stage XII-XIV recipient embryos were described in the specification, and somatic cell chimeras made using Stage X recipient cells were not shown. In the Examiner's opinion, then, the cells can only be described as PGCs in that they contribute to the germline, but have not been definitively established as being pluripotent EG cells in that the applicants have not shown they can be used to make somatic cell chimeras. Applicants respectively traverse this ground for the rejection.

First, Applicants note that this rejection appears to be inconsistent with the Examiner's indication that (3) above is enabling. If the specification enables a method of making germline or somatic cell avians using EG cells isolated from cells isolated from Stage XII-XIV embryos following culturing in the recited growth factors, then it follows that the specification enables the isolation of EG cells from cells isolated from Stage XII-XIV embryos. Nevertheless, Applicants respectfully request reconsideration of this first ground for the rejection, because the specification shows that EG cells were produced by culturing the disclosed PGCs from Stage XII-XIV embryos.

The Examiner is correct to note that transferring the EG cells to Stage X recipient embryos in order to isolate somatic cell chimeras is one way to demonstrate the pluripotency of the EG cells. Applicants disclosed this test at page 22, lines 10-21 of the specification, and agree this is one way to confirm pluripotency. However, this is not the only means to show that EG cells have been generated. Specifically, one way to demonstrate the recovery of EG cells is by staining for EG/ES cell-specific markers. Another method involves showing that the cells differentiate into other cell types when permitted to differentiate *in vitro*.

While the Examiner is correct to point out that some EG/ES cell surface markers are shared with PGCs and thus are not helpful in determining whether PGCs have de-differentiated to the EG cell phenotype, applicants found the MC-480 antibody to be

particularly informative. As discussed on page 42 of the specification, lines 4-22, the MC-480 antibody reacted strongly with mouse EG cells, and with the cells of the present invention after they had been cultured long term (98 days). In contrast, very few fresh PGCs were positive for the MC-480 antigen, and fibroblasts were always negative, suggesting to applicants that the long term culture PGCs of the invention had de-differentiated to EG cells. These results are consistent with those of Matsui et al. (1992), who demonstrated a similar phenomenon with murine primordial germ cells (abstract attached for Examiner's convenience).

Even if the Examiner is not convinced by the MC-480 staining experiment, the fact that the cells appear to differentiate in long term culture should be evidence of their pluripotency. For instance, in the paragraph bridging pages 39-40, the specification describes how after 3 to 5 passages, the cells slow down and become "fibroblast looking." In addition, one cell line, P110596, was established from the isolated PGCs that had a neuronal cell morphology (see page 40, first full paragraph).

Finally, it is known generally in the art that EG cells are isolated from PGCs following in vitro culture, and PGCs need not be used directly from Stage X embryos. For instance, as reported by Shamblott et al. (paper attached), human PGCs give rise to large multicellular colonies yielding EG cells over a period of 7-21 days in culture (see the abstract). Nakatsuji and Chuma (2000) report that mouse PGCs can restart rapid proliferation and transform into pluripotent EG cells even after a period of growth arrest in the beginning stages of sex differentiation (see the abstract). Etches et al. (1996) (abstract attached) report that PGCs obtained from Stage X embryos retain the ability to contribute to both the germline and somatic cell tissues even after being cultured as explanted intact embryos for two more days. According to Wentworth et al. (1989) (abstract attached), PGCs may be isolated from Stage 7 germinal crescents, Stage 17 blood, and Stage 30 gonads, suggesting the PGC phenotype survives migration and development of the gonads. And most importantly, Park and Han (2000) (abstract attached) recently reported the isolation of an EG cell line using PGCs from Stage 28 chicken embryo gonads.

The success of Park and Han in isolating an EG cell line from a Stage 28 chicken embryo supports the applicants' disclosure of the isolation of EG cells from Stage XII-XIV PGCs, as do the other abstracts discussed above that report that PGCs can be isolated from later stages than Stage X embryos. In contrast, the Examiner has presented no evidence to

support his doubting of applicant's disclosure, no evidence to suggest that applicants' cells are not EG cells, and no evidence to suggest that one could not isolate EG cells starting with PGCs from Stage XII-XIV avian embryos.

Thus, given the results reported in the specification with the MC-480 antibody and the evidence that the disclosed PGCs are capable of differentiating following long term culture, it is reasonable to believe that EG cells may be isolated from Stage XII-XIV PGCs as disclosed in the specification without undue experimentation. Furthermore, one could readily screen or test such cells for pluripotency by transferring the cells to a Stage X recipient embryo as described in the specification on page 22, lines 10-21. Accordingly, Applicants respectfully request reconsideration and withdrawal of this ground for the §112, first paragraph rejection, and confirmation that the specification enables the production of EG/ES cells from cells isolated from the blood of Stage XII-XIV embryos.

Second, the Examiner believes that, while the specification enables (2) above, a method of making germline chimeric avians using PGCs isolated from the blood of Stage XII-XIV embryos following culturing in the recited growth factors, and (3) above, a method of making germline or somatic cell avians using EG cells isolated from cells isolated from Stage XII-XIV embryos following culturing in the recited growth factors, it allegedly fails to enable (b) above, using PGCs isolated from the blood of Stage XII-XIV embryos to make either germline or somatic cell embyros. The Examiner's basis for this distinction is that, while PGCs may be used to make germline chimeras, they cannot be used to make somatic cell chimeras unless they can first be dedifferentiated into EG cells. But having isolated EG cells from the disclosed PGCs, the applicants enable the production of germline or somatic cell chimeras using these EG cells.

Applicants understand the distinction the Examiner is trying to make, and have submitted new claims 25 and 26 in an attempt to differentiate these methods. New claims 25 and 26 are similar to claim 14 except that they correlate the making of germline chimeras, and germline or somatic cell chimeras, respectively, with the use of PGCs and EG cells, respectively, in the claimed method. Applicants prefer to continue prosecution of claim 14 as a generic claim that encompasses both embodiments, however, given that claim 14 addresses another novel aspect of the invention, e.g., that the PGCs and EG cells of the invention can be maintained in the absence of a feeder layer. Although this ground for the rejection appears to be in conflict with the first ground discussed above, applicants hope that in reconsidering the

first ground above the Examiner will confirm that the specification does enable a method of making germline and somatic cell chimeras using the EG cells disclosed therein. Nevertheless, claim 25 at the very least should be enabled by the specification taking the comments in the Office Action as a whole.

Next, the Examiner believes that the specification fails to enable (c) stably transfecting EG/ES cells or PGCs, (d) making transgenic avians to isolate proteins or (e) to isolate phenotypically altered transgenic avians, or (g) transfected EG/ES cells. In particular, the Examiner states on page 6 of the Office Action that the specification teaches that stably transfected PGCs have not been obtained (in reference to page 20, line 13 of the specification), and that neither the specification nor the art teach obtaining stably transfected EG or ES cells or making transgenic birds using EG or ES cells comprising an exogenous transgene as claimed. Applicants respectfully traverse this ground for the rejection.

Applicants respectfully submit that it is not true that the art does not teach the production of transfected EG cells and the use thereof to make transgenic birds. In this regard, applicants note that they have submitted arguments in response to this rejection that appear to have gone unanswered. Now that a new non-final action has issued, applicants prior arguments in this regard appear to have been over-looked. Furthermore, the Examiner's reference to Page 20, line 13 of the specification appears to disregard the statement just two lines down on that page, where applicants indicated that although stable transfectants had not yet been obtained, "it is expected that this can be accomplished using known techniques."

To reiterate, applicants respectfully stress that the prior art had not failed in producing chimeric or transgenic avians, as evidenced by the Vick & Simkiss article previously submitted (also see the Allioli abstract attached hereto). Rather, such avians were previously *difficult* to produce due to the inability to culture PGCs for prolonged periods. Thus, the goal of the present invention was not to produce a transgenic or chimeric avian per se as a novel invention. Rather, applicants' goal was to develop a long term culture system for avian PGCs that would *facilitate* the production of transgenic and chimeric avians.

Further, the ability to culture PGCs for prolonged periods not only facilitates the isolation of transfected PGCs, but also facilitates the isolation of transfected EG cells, because EG cell lines are produced as a result of long term culture of PGCs (see specification, page 6, lines 20-29). Facilitation of a transfected EG cell necessarily follows facilitation of

transfected PGCs, because transfected PGCs become transfected EG cells by the culturing process for which the present invention provides.

Thus, the Examiner has rejected claims directed to methods of making transgenic chimeric avians because of deficiencies in the prior art that applicants have in fact overcome. Moreover, the Examiner has rejected claims directed to such methods on the basis that no transgenic chimeric avians were demonstrated in a working example, when in fact such animals had been made before, just with much more difficulty in the absence of the present invention, which provides for long term culture of avian PGCs.

In this regard, the Federal Circuit has long held that 35 USC §112 does not require a specific teaching of that which is already known to one of ordinary skill in the art. Case v. CPC International, Inc., 221 USPQ 196, 201 (Fed. Cir. 1984). Moreover, it is well-established that the "absence of working examples in specification is without significance, since examples are not necessary and even though [they] may provide added useful information, the test is whether an individual possessed of knowledge of one skilled in the art could practice invention without exercise of undue amount of experimentation." Ex Parte Nardi & Simier, 229 USPQ 79, 80 (Bd. App. 1986).

That applicants report that only 1 in 50 PGCs was transfected is not fatal, because the enablement test does not preclude all experimentation, just that which is undue. The fact that some experimentation is necessary to achieve transfection is true for any new cell line or a new vector construct, and in fact was true of the prior art techniques. As evidenced by Vick and Simkiss, persons of ordinary skill were not deterred from making transgenic chimeric avians even though they could not culture PGCs more than a few days, even though they could not confirm or select transfected PGCs before they proceeded with injection of the cells, and even though they had to go through the entire process of hatching the chimeric birds and mating them to see if the transfection enabled germ line transmission. The fact that persons using prior art techniques still sought to make transgenic chimeric birds despite the fact that they did not have the benefit of applicants' culturing method suggests that, even in this uncertain environment, the level of experimentation was not undue. Applicants fail to comprehend, then, how the presently claimed methods of making transgenic chimeric avians would require "undue" experimentation when the claimed methods only make the prior art methods easier, and the level of experimentation was not so inconceivably large to deter prior

researchers from making transgenic chimeric birds in the absence of the benefits to be gained by applicants' invention.

Nevertheless, applicants submitted in the previous Reply filed May 22, 2000, photographs of the EG cells of the present invention expressing a green fluorescence protein (GFP) transgene. These photographs served as substantive evidence that the cells are capable of being transfected notwithstanding the fact that the specification need not disclose that which is known in the art. Furthermore, as discussed in the Reply filed January 2, 2001, it is not clear why expression of a marker gene such as GFP is not a sufficient means to demonstrate that expression of heterologous proteins in general is feasible in the cells and chimeric birds of the invention. This evidence has never been appropriately considered by the Office, therefore, if Applicants need to submit the evidence in the form of a declaration, Applicants would be willing to do so. In this regard, Applicants have attached a copy of the map of the vector employed for these transfection experiments. Reconsideration and withdrawal of this basis for the §112, enablement rejection is respectfully requested.

The Office Action also indicates that claim 3 lacks enablement because the specification does not enable the determination of minimal or maximal amounts of growth factors. In view of the corresponding rejection of claim 3 under 35 U.S.C. §112, second paragraph where the Examiner indicates that there is no antecedent basis for minimal growth factor amounts in claim 1, applicants respectfully submit that the Examiner may be improperly reading claim 3 as being dependent on claim 1. However, claim 3 is dependent on claim 2, and may make more sense if it is read in that context. Claim 2 states the minimal amounts of growth factors, thereby giving meaning to the maximal amounts as they are recited in claim 3. Reconsideration and withdrawal of the rejection is respectfully requested.

Lastly, claim 24 is rejected under 35 U.S.C. §112, first paragraph, because no deposit information is provided for the specific cell line recited in that claim. Applicants respectfully note that this rejection has been rendered moot by the cancellation of claim 24 above.

Next, claims 1-24 were rejected under 35 U.S.C. §112, second paragraph for alleged indefiniteness. In particular, the Examiner submits that the specification fails to give adequate guidance to distinguish between PGCs and EG cells, and that it is unclear whether EG cells are separate from PGCs or whether they have separate characteristics. Further, the Examiner indicates that it is also unclear how EG cells differ from ES cells.

Applicants respectfully submit that the specification adequately describes PGCs and EG cells and also provides teachings as to how the skilled artisan would distinguish between the two. As described in the specification at page 6, lines 20-24 for instance, EG cells are isolated from PGCs following *in vitro* culture. PGCs are cells that migrate to the gonads during embryonic development and eventually commit to a sexually differentiated path, resulting in the germ cells or sex cells. If isolated prior to germ cell development and placed in culture, it is possible to isolate "dedifferentiated" embryonic germ (EG) cells from PGCs, wherein such EG cells have regained their pluripotency and their potential to differentiate into other cell types. This is consistent with what it is known and reported by others in the art (see Shamblott et al., attached hereto), and the specification adequately describes this phenomenon (see page 42, for instance).

The specification also provides several ways to determine whether the PGCs of the invention have dedifferentiated into EG cells. One way is to transfer the cells into a recipient Stage X embryo and see if the cells can be used to make either germline or somatic cell chimeras (page 22, lines 10-21). Another way is to see if the cells express surface markers consistent with a pluripotent phenotype (e.g., screen for the MC-480 antigen, for instance). Another way is to see if the cells differentiate into other cell types when permitted to grow without passage in culture (page 39, lines 20-23).

With regard to ES and EG cells, these cells are both pluripotent cells that have the ability to differentiate into all different cell types. The only distinction is that, while ES cells are derived from the inner cell mass of a blastocyst for mammals and the blastoderm disc in chickens, EG cells are generally derived by "de-differentiating" primordial germ cells in culture after they are taken from the genital ridge of an embryo. U.S. Patent No. 6,245,566 provides a useful discussion to supplement this discussion. In this regard, the Examiner makes a fundamental error in the Office Action when he says that Applicants have failed to enable the isolation of EG/ES cells "from the blood" of stage 12-14 chicken embryos (see page 3), in that EG cells are not isolated from the blood; they are isolated from cultured PGCs. Applicants hope these comments and those submitted above in response to the §112, first rejection will help resolve the clarity issues raised in the Office Action.

As mentioned briefly above, claim 3 was rejected under §112, second paragraph, because the phrase "maximal amounts of said growth factors range from about two times to one hundred times said minimum amounts" is unclear, and that "said minimum amounts"

lacks antecedent basis in claim 1. In applicants' file, claim 3 is dependent on claim 2, which provides the necessary antecedent basis. Furthermore, claim 2 provides the requisite minimal amounts that create the frame of reference for the way the maximal amounts are recited in claim 3. Reconsideration and withdrawal of these rejections under §112, second paragraph is respectfully requested.

Claims 1, 3-6, 21 and 22 were rejected under 35 U.S.C. §102(b) as being allegedly anticipated by Chang et al. Applicants respectfully submit that Chang does not appear to be an appropriate §102 reference because Chang does not teach all the limitations of the claimed invention, i.e., the addition of SCF. The Examiner avoids this problem by arguing that SCF does not appear to be required, yet applicants have stressed that in their experience, SCF was indeed required. Nevertheless, claim 1 has been amended to require the absence of a feeder layer, as do the newly submitted claims. Furthermore, the newly submitted claims require culturing for more than fourteen days, which is significantly longer than the five day culturing period in Chang et al. Thus, this rejection should be rendered moot as to claims 1, 3-6, 21 and 22, and should not be applicable to any of the newly submitted claims.

Claims 1, 3-11, 14-16 and 20-22 are rejected under 35 U.S.C. §102(b) as being allegedly anticipated by Pain as evidenced by Simkiss. The Examiner alleges that Pain teaches the isolation of cells from the blastoderm of a Stage X chicken embryo, and the culturing of these cells for more than 160 days in the presence of bFGF, IGF, SCF and LIF without feeder cells. Applicants respectfully submit that this is an erroneous reading of the reference. Applicant reviewed all the citations in the document noted by the Examiner where it is alleged to disclose the absence of feeder cells in the culturing medium. Generally, these citations stated that feeder cells <u>were</u> in fact employed for the long term culture. For instance, the noted sentence at page 2345, col. 2, line 10, states "The culture conditions, <u>which included the use of mouse embryonic feeder cells</u> and the inclusion of LIF, IL-11, SCF, bFGF, IGF-1 and ARMA in the medium, facilitated the proliferation of cells with an undifferentiated phenotype during more than 35 passages . . .". The one passage referenced correctly in the Office Action that did report the culture of cells without a feeder layer was on page 2341, col.2, paragraph 4, however, this section concerned the testing of the effect of individual growth factors on the growth of the cells, <u>not</u> the long term passage of the cells in an undifferentiated state. Thus, it appears that Pain et al. does not teach the long term maintenance of PGCs in the absence of a feeder layer as alleged in the Office Action.

Furthermore, as discussed in the Reply filed May 22, 2000, Pain et al. did not use the mixture of the growth factors recited in the claims. Pain et al. also employed IL-11 and antiretinoic acid monoclonal antibody, which prevents differentiation of the cells. Thus, even if the skilled artisan could reasonably rely on Pain et al. to arrive at a medium containing the four recited growth factors for isolating and cultivating EG cells in the absence of a feeder layer, one would also believe it necessary based on Pain et al. to add IL-11 and antiretinoic acid monoclonal antibody in order to prevent cell differentiation. In contrast, applicants have discovered that avian PGCs may be maintained in long term culture and used to produce EG cells with only the four recited growth factors being required (other than basic essentials).

Nevertheless, despite the differences in growth factors employed by Pain and applicants, Applicants note that claims 1 and 14 have been amended above to require the absence of a feeder layer. Since Pain does not teach the absence of a feeder layer in the context of these claims, Pain is not prior art to the claimed invention. Nor is Pain prior art to the newly submitted claims, because the newly submitted claims require PGCs from a Stage XII-XIV embryo, or EG cells isolated therefrom. Pain uses cells from a Stage X embryo, and therefore cannot be a §102 reference against these claims. Furthermore, such claims are not rendered obvious in view of Pain, seeing as it is unexpected that applicants accomplished the claimed methods using cells derived from Stage XII-XIV embryos as argued by the Examiner in the rejection under 35 U.S.C. §112, first paragraph. Thus, reconsideration and withdrawal of the rejection based on Pain is respectfully requested.

Claims 1, 21 and 22 were rejected under 35 U.S.C. §102(e) as being allegedly anticipated by Pettitte, because the ES cells of Pettitte are isolated from Stage IX-XIV embryos, have an ES cell phenotype, and are cultured in the presence of LIF. Although Pettitte uses a feeder layer, the Examiner indicates that the claims do not require the absence of a feeder layer. Applicants respectfully note that culturing in the presence of LIF does not appear to meet all the elements of the claimed invention since LIF is not the only growth factor required by the claims. Therefore, Pettitte does not appear to be a proper §102 reference. Nevertheless, applicants have amended the claims to require feeder layer, so this rejection appears to be rendered moot. Reconsideration and withdrawal is respectfully requested.

Finally, claims 1 and 2 were rejected under 35 U.S.C. §103(a) as being allegedly unpatentable over Pain and Simkiss, because in the Examiner's opinion, it would be obvious

to vary the culture conditions to achieve the specific amounts of growth factors recited in claim 2 because Pain teaches to vary the culture conditions. Applicants respectfully submit that it could not be obvious to achieve these specific growth factor concentrations by optimization because Pain does not use this growth factor combination to begin with. Again, as argued above, Pain also uses IL-11 and antiretinoic acid monoclonal antibody, which prevents differentiation of the cells. Furthermore, Pain uses feeder cells in the long term culturing of the disclosed cells. Feeder cells are an unwelcome source of contamination with respect to the generating of chimeric animals that can affect the efficiency of recovery of such animals. It would have been truly unexpected at the time the present invention was made, and even now, that PGCs could be made to proliferate for prolonged periods in culture in the absence of feeder cells. Thus, reconsideration and withdrawal of the §103(a) rejection based on Pain et al. and Simkiss is respectfully requested.

Applicants acknowledge the obvious-type double patenting rejections set forth in the Office Action, and request that they be held in abeyance until the indication of allowable subject matter.

This Reply is fully responsive to the Office Action dated March 19, 2001. In view of the foregoing, the claims are now believed to be in form for allowance, and such action is hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, he is kindly requested to contact the undersigned at the telephone number listed below.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached Appendix is captioned **"Version with markings to show changes made"**.

Respectfully submitted,

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APPENDIX: VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims

Claims 1 and 14 were amended as follows:

1. (Twice Amended) A culturing method which provides for the production of avian PGC and germ (EG) cells comprising the following steps:

(i) isolating primordial germ cells (PGCs) from a desired avian; [and]

(ii) culturing said [primordial germ cells] PGCs in a culture medium containing at least the following growth factors [contained] in amounts sufficient to maintain said PGCs for prolonged periods in tissue culture:

(1) leukemia inhibitory factor (LIF),

(2) basic fibroblast growth factor (bFGF),

(3) stem cell factor (SCF) and

(4) insulin-like growth factor (IGF),

for [prolonged] a time period sufficient to produce a culture having a compact multilayer like appearance whereby said culturing [may be] is performed in the absence of a feeder layer; and

(iii) identifying EG cells contained therein.

14. (Twice Amended) An improved method of producing chimeric avians which comprises:

(i) isolating primordial germ cells (PGCs) from an avian;

(ii) maintaining such PGCs in a tissue culture medium containing at least the following growth factors;

(1) leukemia inhibitory factor (LIF),

(2) basic fibroblast growth factor (bFGF),

(3) stem cell factor (SCF) and

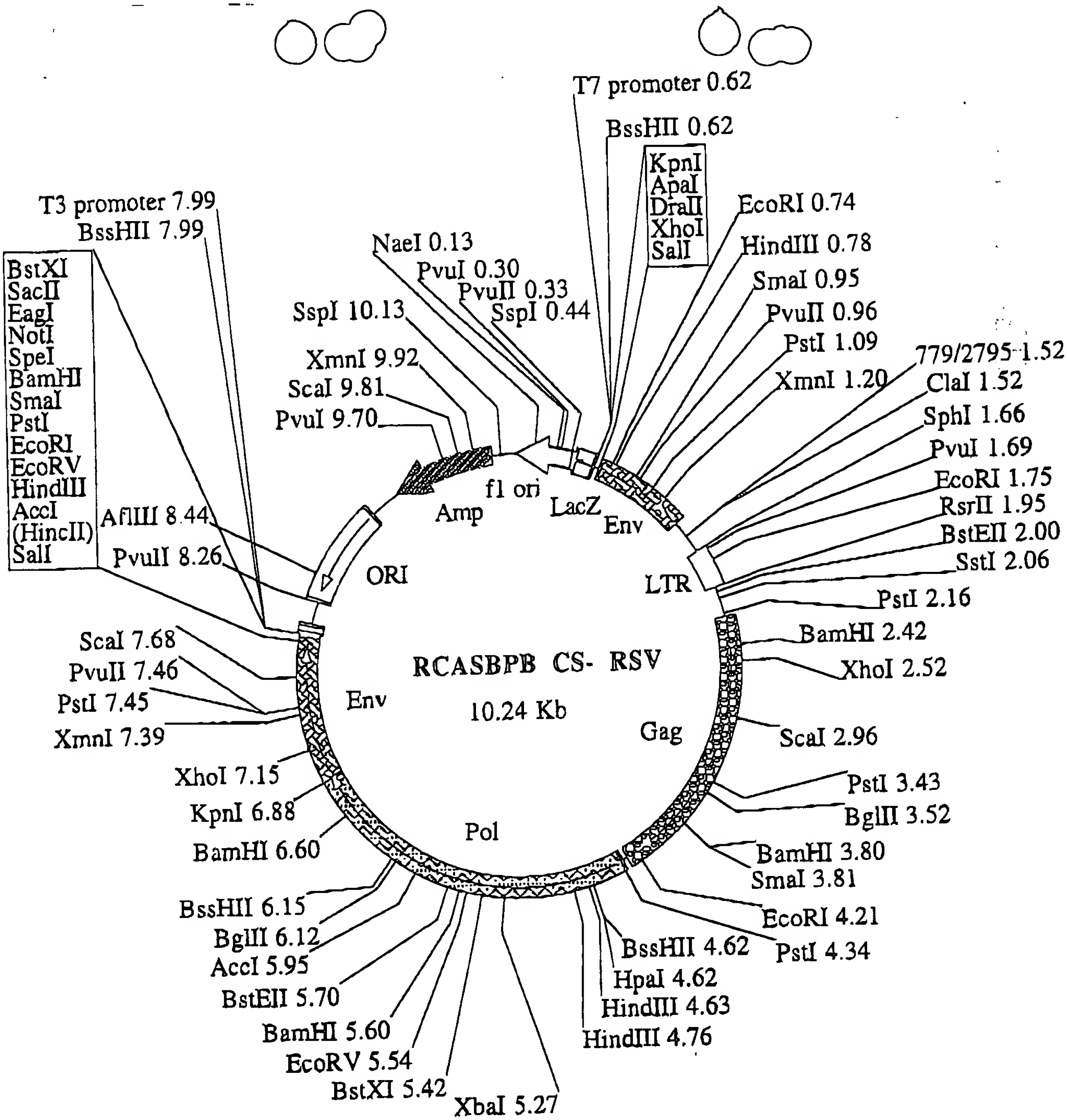
(5) insulin-like growth factor (IGF)

for a sufficient time to produce embryonic germ (EG) cells whereby said culture [may be] is maintained in the absence of a feeder layer;

LA8

(iii) transferring said EG cells into a recipient avian embryo; and

(iv) selecting for chimeric avians which have the desired [PGC] EG phenotype.



Plasmid name: RCASBPB CS- RSV
Plasmid size: 10.24 kb
Constructed by: Kirstin Dohrer and Brian Ruis
Construction date: 5/1/96
Comments/References: RCASBPB CS- RAV0, Hyb II, Hyb III, and RSV have the same sequences except for their respective LTR's. Only and approximate map

BASIC VIRAL VECTOR USED TO TRANSFECT PGCs, PROVIDED BY Dr CONKLIN'S LAB
LAC Z GENE HAS BEEN REPLACED WITH GFP GENE

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1: Dev Biol 1994 Sep;165(1):30-7

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Use of retroviral vectors to introduce and express the beta-galactosidase marker gene in cultured chicken primordial gerr cells.

Allioli N, Thomas JL, Chebloune Y, Nigon VM, Verdier G, Legras C.

Centre de Genetique Moleculaire et Cellulaire, CNRS UMR106, INRA LA810, Universite Claude Bernard Lyon-I, Villeurbanne, France.

Three methods of isolating primordial germ cells (PGCs) from gonads of 5-day-old chick embryos were compared. PGCs were then cultured in vitro in DMEM/F12 medium containing 10% fetal calf serum. BrdU incorporation showed that at least 10% of the PGC population were dividing, under our culture conditions, during the 2nd day of in vitro culture. During this cultur period, PGCs were exposed to avian leukosis sarcoma virus-based retrovira vector pseudotyped with subgroup A envelope, carrying the LacZ reporter gene. X-Gal staining showed that PGCs were permissive to infection, with more than 50% of PGCs expressing the beta-Gal protein. These data repres the first demonstration that PGCs, isolated from gonads of 5-day-old chick embryos, are able to divide in vitro and that it is possible to introduce and express exogenous DNA in chick PGCs maintained in vitro.

PMID: 8088448 [PubMed - indexed for MEDLINE]

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1: Cell 1992 Sep 4;70(5):841-7

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Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture.

Matsui Y, Zsebo K, Hogan BL.

Department of Cell Biology Vanderbilt University Medical School Nashvil] Tennessee 37232.

Steel factor (SF) and LIF (leukemia inhibitory factor) synergistically promo the proliferation and survival of mouse primordial germ cells (PGCs), but o for a limited time period in culture. We show here that addition of bFGF to cultures in the presence of membrane-associated SF and LIF enhances the growth of PGCs and allows their continued proliferation beyond the time when they normally stop dividing in vivo. They form colonies of densely packed, alkaline phosphatase-positive, SSEA-1-positive cells resembling undifferentiated embryonic stem (ES) cells in morphology. These cultures c be maintained on feeder layers for at least 20 passages, and under appropria conditions give rise to embryoid bodies and to multiple differentiated cell phenotypes in monolayer culture and in tumors in nude mice. PGC-derived cells can also contribute to chimeras when injected into host blastocysts. Th long-term culture of PGCs and their reprogramming to pluripotential ES ce has important implications for germ cell biology and the induction of teratocarcinomas.

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Developmental Biology

Derivation of pluripotent stem cells from cultured human primordial germ cells

(*alkaline phosphatase/embryoid body/embryonic stem cell/embryonic germ cell*)

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ABSTRACT

Human pluripotent stem cells would be invaluable for *in vitro* studies of aspects of human embryogenesis. With the goal of establishing pluripotent stem cell lines, gonadal ridges and mesenteries containing primordial germ cells (PGCs, 5-9 weeks postfertilization) were cultured on mouse STO fibroblast feeder layers in the presence of human recombinant leukemia inhibitory factor, human recombinant basic fibroblast growth factor, and forskolin. Initially, single PGCs in culture were visualized by alkaline phosphatase activity staining. Over a period of 7-21 days, PGCs gave rise to large multicellular colonies resembling those of mouse pluripotent stem cells termed embryonic stem and embryonic germ (EG) cells. Throughout the culture period most cells within the colonies continued to be alkaline phosphatase-positive and tested positive against a panel of five immunological markers (SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81) that have been used routinely to characterize embryonic stem and EG cells. The cultured cells have been continuously passaged and found to be karyotypically normal and stable. Both XX and XY cell cultures have been obtained. Immunohistochemical analysis of embryoid bodies

collected from these cultures revealed a wide variety of differentiated cell types, including derivatives of all three embryonic germ layers. Based on their origin and demonstrated properties, these human PGC-derived cultures meet the criteria for pluripotent stem cells and most closely resemble EG cells.

INTRODUCTION

Pluripotent stem cells have been derived from two embryonic sources. Embryonic stem (ES) cells are derived from the inner cell mass of preimplantation embryos (1, 2), and embryonic germ (EG) cells are derived from primordial germ cells (PGCs) (3, 4). Both ES and EG cells are pluripotent and demonstrate germ-line transmission in experimentally produced chimeras (5, 6). Mouse ES and EG cells share several morphological characteristics such as high levels of intracellular alkaline phosphatase (AP), and presentation of specific cell surface glycolipids (7, 8) and glycoproteins (9). These properties are characteristic of, but not specific for, pluripotent stem cells. Other important characteristics include growth as multicellular colonies, normal and stable karyotypes, the ability to be continuously passaged, and the capability to differentiate into cells derived from all three embryonic germ layers. Pluripotent stem cell lines that share most of these characteristics also have been reported for chicken (10), mink (11), hamster (12), pig (13, 14), rhesus monkey (15), and common marmoset (16).

The pluripotency of ES and EG cells can be demonstrated *in vitro* and *in vivo*. Embryoid bodies (EBs) are differentiated cell aggregates first described as arising in human (17) and mouse (18-20) teratomas and teratocarcinomas. These aggregates range from a cluster of pluripotent stem cells enclosed by a layer of endoderm to complex structures closely resembling an embryo during early development. EBs from mouse pluripotent stem cells grown on feeder layers or in suspension may contain a variety of cell types. This property has been used as evidence of cell pluripotency (1, 21) and as a source of differentiating cells. With the proper combinations of growth and differentiation factors, mouse ES (22, 23) and EG (S.W., unpublished results) cultures can generate cells of the hematopoietic lineage and cardiomyocytes (24, 25). In addition, mouse ES cells have been used to generate *in vitro* cultures of neurons (26), skeletal muscle (27), and vascular endothelial cells (28). ES and EG cells from some species can form teratocarcinomas when injected into histocompatible or immunologically compromised mice. This property alone may not be a definitive test of stem cell pluripotency, as it has been demonstrated that rat and mouse visceral (yolk sac) endoderm are capable of forming highly differentiated teratomas containing cells of all three embryonic germ layers (29, 30). Perhaps the most definitive *in vivo* test of developmental potential would be a demonstrated contribution to all cell lineages in a chimeric animal, but this test is not practical or possible for all species and cannot be done with human cells.

We report here the establishment of cultures from human PGCs. These cultures have morphological, immunohistochemical, and karyotypic features consistent with those of previously described pluripotent stem cells and have a demonstrated ability to differentiate *in vitro* into derivatives of the

three embryonic germ layers.

MATERIALS AND METHODS

Collection of Tissue, Establishment, and Maintenance of Cultures. Gonadal ridges and mesenteries of 5- to 9-week postfertilization human embryos (obtained as a result of therapeutic termination of pregnancy by using a protocol approved by the Joint Committee on Clinical Investigation of the Johns Hopkins University School of Medicine) were mechanically disaggregated then incubated in 0.05% trypsin-0.5 mM EDTA (GIBCO/BRL) or 0.25% trypsin at 37°C for 5-10 min, or incubated in a combination of 0.01% hyaluronidase type V (Sigma), 0.1% collagenase type IV (Sigma), and 0.002% DNase type I (Sigma) at 37°C for 2 hr (31). Cells initially were cultured and subsequently passaged on a mouse STO fibroblast feeder layer mitotically inactivated with 5,000 rads (1 rad = 0.01 Gy) γ-radiation. Cells were grown in DMEM (GIBCO/BRL) supplemented with 15% fetal bovine serum (HyClone), 0.1 mM nonessential amino acids (GIBCO/BRL), 0.1 mM 2-mercaptoethanol (Sigma), 2 mM glutamine (GIBCO/BRL), 1 mM sodium pyruvate (GIBCO/BRL), 100 units/ml of penicillin (GIBCO/BRL), 100 μg/ml of streptomycin (GIBCO/BRL), 1,000 units/ml of human recombinant leukemia inhibitory factor (hrLIF, Genzyme), 1 ng/ml of human recombinant basic fibroblast growth factor (hrbFGF, Genzyme), and 10 μM forskolin (Sigma). Cultures were grown in 5% or 8% CO_2, 95% humidity and were routinely passaged every 7 days after disaggregation with 0.05% trypsin/0.53 mM EDTA (GIBCO/BRL) or 0.25% trypsin at 37°C for 5-10 min.

Initial Characterization. Cells were fixed for detection of AP activity in 66% acetone/3% formaldehyde and then stained with naphthol/FRV-alkaline AP substrate (Sigma). For immunocytochemistry, cells were fixed in 3% paraformaldehyde in Dulbecco's PBS (GIBCO/BRL). Cell surface glycolipid- and glycoprotein-specific mAbs were used at 1:15 to 1:50 dilution. MC480 (SSEA-1), MC631 (SSEA-3), and MC813-70 (SSEA-4) antibodies were supplied by the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City). TRA-1-60 and TRA-1-81 were a gift of Peter Andrews (University of Sheffield, U.K.). Antibodies were detected by using biotinylated anti-mouse secondary antibody, strepavidin-conjugated horseradish peroxidase, and 3-amino-9-ethylcarbazole chromagen (BioGenex). Cells prepared for cytogenetic analysis were incubated in growth media with 0.1 μg/ml of Colcemid for 3-4 hr, trypsinized, resuspended in 0.075 M KCl, and incubated for 20 min at 37°C, then fixed in 3:1 methanol/acetic acid.

Immunohistochemistry. EBs were collected from cultures and either immediately embedded or replated into single wells of a 96-well tissue culture plate and cultured for 14 days in the absence of hrLIF, hrbFGF, and forskolin, before embedding. For embedding, EBs were placed into a small drop of molten 1% low melting point agarose (FMC) prepared in PBS, and cooled to 42°C. Solidified agarose containing EBs then were fixed in 3% paraformaldehyde in PBS and embedded in paraffin. Individual 6-μm sections were placed on slides (ProbeOn Plus, Fisher Scientific) and

immunohistochemical analysis was carried out by using a BioTek-Tech Mate 1000 automated stainer (Ventana-BioTek Solutions, Tucson, AZ). Antibodies used on paraffin sections included: HHF35 (muscle-specific actin, Dako), M 760 (desmin, Dako), CD34 (Immunotech, Luminy, France), Z 311, (S-100, Dako), sm311 (pan-neurofilament, Sternberger Monoclonals, Baltimore, MD), A 008 (alpha-1-fetoprotein, Dako), CKERAE1/AE3 (pan-cytokeratin, Boehringer Mannheim), OV-TL 12/30 (cytokeratin 7, Dako), and K_s20.8 (cytokeratin 20, Dako). Primary antibodies were detected by using biotinylated anti-rabbit or anti-mouse secondary antibody, strepavidin-conjugated horseradish peroxidase, and diaminobenzidine chromagen (Ventana-BioTek Solutions). Slides were counterstained with hematoxylin.

RESULTS

Of 38 human PGC cultures initiated, 36 (≈95%) demonstrated morphological, biochemical, and/or immunocytochemical characteristics consistent with previously characterized pluripotent stem cell lines. High levels of AP activity are associated with ES and EG cultures, as well as with PGCs *in vivo* (32). As seen in Fig. 1, cells that closely resemble individual stationary and migratory mouse PGCs (33) are readily detected in the initial plating of human PGCs. In the presence of an irradiated STO cell feeder layer, hrbFGF, forskolin, and hrLIF, solitary PGCs gave rise to large tightly compacted multicellular colonies resembling early passage mouse ES and EG cell colonies (Fig. 2). This morphology is in contrast to the flattened and loosely associated colonies typical of human embryonal carcinoma (34) and rhesus ES cells (15). The conversion efficiency from PGCs, which have limited *in vitro* survival and proliferative capacity in the mouse (35, 36), to cells that can be passaged at least 20 times was variable and did not depend on the embryonic stage or sex of the embryo. Generation and passage of derived cultures were less successful when mouse embryo fibroblasts, human fetal fibroblasts, or gelatin-coated tissue culture dishes were substituted for STO cells, or when hrLIF or hrbFGF were withdrawn. Unlike mouse pluripotent stem cells (ES and EG), these human cells were more resistant to disaggregation by trypsin/EDTA-based reagents.

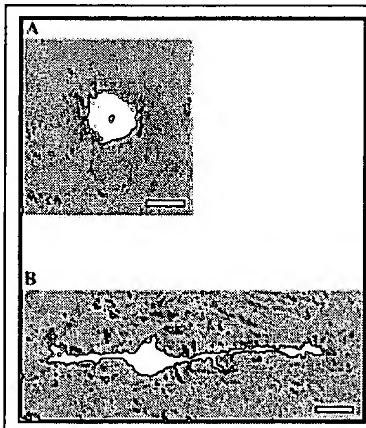

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Fig. 1. AP activity of individual human PGCs in culture. (*A*) Stationary and (*B*) migratory PGCs in a primary culture, growing on a feeder layer of mitotically inactivated mouse STO fibroblasts. (Bars represent 10 μm.)

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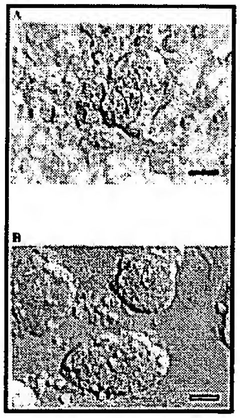

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Fig. 2. Colony morphology. (*A*) Human PGC-derived cell colony growing on a feeder layer of mitotically inactivated mouse STO fibroblasts. (*B*) Mouse ES colony growing on a feeder layer of mitotically inactivated mouse fibroblasts. Hoffman modulation optics. (Bars represent 100 μm.)

Like ES and EG cells from other species, the human PGC-derived cells possess high levels of AP activity (Fig. 3*A*). The percentage of visible AP-positive cells within a colony varied from ≈20% to >90%. Human PGC-derived cells were further characterized with a bank of five mAbs: SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 (7, 8). As seen in Fig. 3 *B-F*, colonies stained strongly for four of the five antibodies, whereas colonies stained with the secondary antibody alone gave no signal (data not shown). The antibody recognizing SSEA-3 antigen stained the cells inconsistently and weakly. As with the results of AP staining, the percentage of cells within a colony that stained positive was variable.

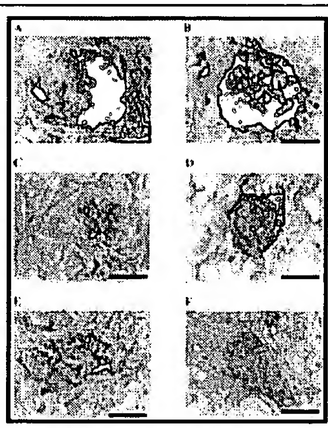

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Fig. 3. Expression of cell surface markers by human PGC-derived cell colonies. (*A*) AP. (*B*) SSEA-1. (*C*) SSEA-3. (*D*) SSEA-4. (*E*) TRA-1-60. (*F*) TRA-1-81. (Bars represent 100 μm.)

Karyotypic analyses carried out at passage 8-10 (60-70 days in culture) indicated apparently normal human chromosomes at the 300 band level of resolution (37). Of five different cultures examined, three were XX and two were XY. Karyotypes of each sex are shown in Fig. 4.

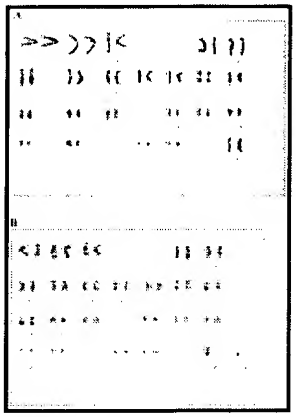

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Fig. 4. Karyotype of human PGC-derived cell cultures. (*A*) XX, eight passages. (*B*) XY, 10 passages.

In the human PGC-derived cultures, a small percentage (≈1-20%) of colonies spontaneously generate EBs in the presence of hrLIF. Table 1 indicates antibody reactivity of the EBs. Immunohistochemical analysis of serial sections represents a sampling of the cells within an EB so the absence of a particular marker is not significant. However, most of the markers were represented in all of the EBs.

Table 1. Immunohistochemical analysis of six human embryoid bodies

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Immunohistochemical analysis of the EBs demonstrated that PGC-derived cells can differentiate into a variety of cell types, including derivatives of the three embryonic germ layers. Three distinct mesodermal derivatives were seen. These were antimuscle specific actin-reactive myocytes with prominent eccentric nuclei and cytoplasmic filaments (Fig. 5*A*), antidesmin-reactive mesenchymal cells (Fig. 5*B*), and anti-CD34-reactive vascular endothelium (Fig. 5*C*). Ectodermal derivatives include cells suggestive of neuroepithelia with nuclear localized anti-S-100-reactivity (Fig. 5 *D* and *E*) and antineurofilament-reactive cells (Fig. 5*F*). Endodermal derivatives include anti-α-1-fetoprotein-reactive cells, which appear within the interior of some EBs as well as form the exterior layer (Fig. 5*G*). Several types of anticytokeratin-reactive epithelia were seen, including nests of relatively undifferentiated cells (Fig. 5*H*) and simple cuboidal epithelial layers (Fig. 5*I*).

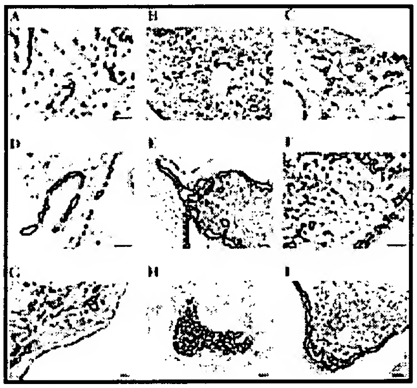

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Fig. 5. Immunohistological analysis of human EB sections. EB culture designation, antibody epitope, and objective power are as follows: (*A*) BF1, muscle-specific actin, ×100. Arrow indicates a cell with eccentric nuclei and cytoplasmic filaments. (*B*) RI5, desmin, ×60. (*C*) BF1, CD34, ×60. (*D*) BF1, S-100, ×100. (*E*) RI5, S-100, ×60. (*F*) RI, pan-neurofilament, ×100. (*G*) BF1, α-1-fetoprotein, ×60. (*H*) BF1, pan-cytokeratin, ×60. (*I*) RI5, cytokeratin 7, ×60. (Bars represent 20 μm.)

DISCUSSION

The human cultures described here satisfy the criteria used to define pluripotent stem cells. These include presentation of a series of markers commonly used to identify pluripotent stem cells, morphological similarity to mouse ES and EG cells, normal and stable karyotype maintained over at least 10 passages, and demonstrated ability to differentiate into a wide variety of cell types.

The histological profile of these human cells (AP+, SSEA-1+, SSEA-3+, SSEA-4+, TRA-1-60+, and TRA-1-81+) differs from undifferentiated human embryonal carcinoma (EC) and rhesus ES cells, which are SSEA-1 negative (15, 38). The fact that differentiation of the human EC line NTERA2 leads to increased expression of SSEA-1 may suggest that this marker is indicative of differentiation in the human PGC-derived cultures. However, NTERA2 differentiation is accompanied by the loss of the other markers (39, 40), which we do not observe. A second possibility is that SSEA-1 reactivity reflects an intrinsic difference between the relatively flat and loosely associated human EC and rhesus ES colonies and the multilayered and tightly compacted colonies formed by mouse ES and EG cells that are SSEA-1 positive.

Human cell cultures derived and grown as described have been continuously maintained for more than 20 passages. Maintaining high colony density and derivation of clonal cell lines is complicated by the difficulties associated with disaggregation of colonies to single cells. The highly compacted nature of these colonies suggests strong cell-cell adhesion. These interactions are notably more resistant to trypsin than mouse ES and EG colonies. Alternative disaggregation enzymes are currently under investigation.

The most useful and important property of these cells is their ability to differentiate *in vitro* into ectodermal, endodermal, and mesodermal derivatives. As with mouse ES and EG cells, human

pluripotent stem cells can form EBs. These structures appear to recapitulate the normal developmental processes of early embryonic stages and promote the cell-cell interaction required for cell differentiation. Typically, mouse EBs are surrounded by a layer of visceral or parietal endoderm and contain a heterogeneous mixture of cell types. Morphologically, human EBs resemble those generated from mouse ES and EG cultures. Many have an outer layer that stains positive for α-1-fetoprotein (Fig. 5*G*) although the constituent cells of this layer do not always resemble those seen in mouse EBs. Identification of cell derivatives of the three embryonic germ layers in human EBs suggests that these cells are pluripotent and are capable of *in vitro* differentiation.

Although the properties of the cultures described in this paper are consistent with those of pluripotent stem cells, the cultures have a lower plating efficiency than most mouse EG and ES cell cultures, which may reflect difficulties associated with complete cell disaggregation. Although many of the PGC-derived cultures have been passaged 20-25 times, the immortality of these cells remains to be demonstrated.

Human pluripotent stem cells, with their potential to differentiate into a wide variety of cell types in culture, would be invaluable for studies of some aspects of human embryogenesis and for transplantation therapies. They may serve to define the culture conditions and differential gene expression necessary for cell-type-specific differentiation and for the isolation of lineage-restricted stem cells that could serve as a source of cells for transplantation. Genetic modification of these pluripotent stem cells may allow the generation of universal donor cells or cells that have been customized to meet individual requirements. Clearly, these goals warrant investigations on the isolation, study, and use of human pluripotent stem cells.

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ABBREVIATIONS

AP, alkaline phosphatase; EG, embryonic germ; ES, embryonic stem; PGC, primordial germ cell; EB, embryoid body; hrLIF, human recombinant leukemia inhibitory factor; hrbFGF, human recombinant basic fibroblast growth factor.

FOOTNOTES

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Differentiation of mouse primordial germ cells into female or male germ cells.

Nakatsuji N, Chuma S.

Department of Development and Differentiation, Institute for Frontier Medi Sciences, Kyoto University, Japan. nnakatsu@frontier.kyoto-u.ac.jp

Mouse primordial germ cells (PGCs) migrate from the base of the allantois the genital ridge. They proliferate both during migration and after their arri until initiation of the sex-differentiation of fetal gonads. Then, PGCs enter i the prophase of the first meiotic division in the ovary to become oocytes, while those in the testis become mitotically arrested to become prospermatogonia. Growth regulation of mouse PGCs has been studied by culturing them on feeder cells. They show a limited period of proliferation i vitro and go into growth arrest, which is in good correlation with their developmental changes in vivo. However, in the presence of multiple grow signals, PGCs can restart rapid proliferation and transform into pluripotent embryonic germ (EG) cells. Observation of ectopic germ cells and studies c reaggregate cultures suggested that both male and female PGCs show cell-autonomous entry into meiosis and differentiation into oocytes if they were apart from the male gonadal environments. Recently, we developed a two-dimensional dispersed culture system in which we can examine transition from the mitotic PGCs into the leptotene stage of the first meiotic division. Such entry into meiosis seems to be programmed in PGCs before reaching t genital ridges and unless it is inhibited by putative signals from the testicul somatic cells.

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1: Poult Sci 1989 Jul;68(7):999-1010 Related Articles, Bo

Manipulation of avian primordial germ cells and gonadal differentiation.

Wentworth BC, Tsai H, Hallett JH, Gonzales DS, Rajcic-Spasojevic G.

Department of Poultry Science, University of Wisconsin, Madison 53706.

The authors hypothesized that donor primordial germ cells (PGC) are usefu vehicles of gene transfer in birds. The PGC have been identified in the blast prior to incubation. They have been isolated from the Stage 7 germinal crescents, Stage 17 blood, and Stage 30 gonads. The percentages of PGC in these three embryonic sources were 2, .003 and 1.5%, respectively. The isolated PGC have been used as antigens to produce specific antibodies that have served as major tools in identification, isolation, and forming enriched vitro cultures of PGC. Sterile hosts for normal donor PGC have been induce by use of ultraviolet irradiation, treatment with the chemical busulfan, and crossing rooster with female quail to form a sterile host termed "quickens." Micromanipulator technology, along with refinement of techniques to avoic trauma, will allow 80% hatchability. Integration of all the above biotechnol has led to successful use of PGC as vehicles of gene transfer and productioi autogenic quail.

Publication Types:
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WILEY InterScience

Derivation and characterization of pluripotent embryonic ger cells in chicken.

Park TS, Han JY.

School of Agricultural Biotechnology, Seoul National University, Suwon, Korea.

Embryonic germ (EG) cell lines established from primordial germ cells (PGCs) are undifferentiated and pluripotent stem cells. To date, EG cells w proven germ-line transmission have been completely established only in the mouse with embryonic stem (ES) cells. We isolated PGCs from 5.5-day-olc (stage 28) chicken embryonic gonads and established a putative chicken EC cell line with EG culture medium supplemented with stem cell factor (SCF) leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), interleukin-11 (IL-11), and insulin-like growth factor-I (IGF-I). These cells grew continuously for ten passages (4 months) on a feeder layer of mitotica active chicken embryonic fibroblasts. After several passages, these cells we characterized by screening with the periodic acid-Schiff reaction, anti-SSE antibody, and a proliferation assay. The chicken EG cells maintained characteristics of gonadal PGCs and undifferentiated stem cells. When cultured in suspension, the chicken EG cells successfully formed an embry body and differentiated into a variety of cell types. The chicken EG cells w injected into stage X blastodermal layer and produced chimeric chickens wi various differentiated tissues derived from the EG cells. Chicken EG cells v be useful for the production of transgenic chickens and for studies of germ differentiation and genomic imprinting.

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1: Mol Reprod Dev 1996 Nov;45(3):291-8

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Contributions to somatic and germline lineages of chicken blastodermal cells maintained in culture.

Etches RJ, Clark ME, Toner A, Liu G, Gibbins AM.

Department of Animal & Poultry Science, University of Guelph, Ontario, Canada.

Chicken blastodermal cells were cultured for 48 hr as explanted intact embryos, as dispersed cells in a monolayer, or with a confluent layer of mou fibroblasts. The cells were then dispersed and injected into stage X (E-G&K recipient embryos that were exposed to 600 rads of irradiation from a 60Co source. Regardless of the conditions in which the cells were cultured, chime with contributions to both somatic tissues and the germline were observed. When blastodermal cells were co-cultured with mouse embryonic fibroblas significantly more somatic chimeras were observed and the proportion of feather follicles derived from donor cells was increased relative to that observed following the injection of cells derived from explanted embryos o monolayer cultures. Culture of blastodermal cells in any of the systems, however, yielded fewer chimeras that exhibited reduced contributions to somatic tissues in comparison to the frequency and extent of somatic chimerism observed following injection of freshly prepared cells. Contributions to the germline were observed at an equal frequency regardle of the conditions of culture, but were significantly reduced in comparison tc the frequency and rate of germline transmission following injection of cells obtained directly from stage X (E-G&K) embryos. These data demonstrate that some cells retain the ability to contribute to germline and somatic tissu after 48 hr in culture and that the ability to contribute to the somatic and germline lineages is not retained equally.

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